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GRAB: A Physiologic Guanine Nucleotide Exchange Factor for Rab3A, which Interacts with Inositol Hexakisphosphate Kinase

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Summary

Diphosphoinositol-pentakisphosphate (InsP7) and bis-diphosphoinositol tetrakisphosphate (InsP8) possess pyrophosphate bonds. InsP7 is formed from inositol hexakisphosphate (InsP6) by recently identified InsP6 kinases designated InsP6K1 and InsP6K2. We now report the identification, cloning, and characterization of a novel protein, GRAB (*g*uanine nucleotide exchange factor for *Rab*3A), which interacts with both InsP6K1 and Rab3A, a Ras-like GTPase that regulates synaptic vesicle exocytosis. GRAB is a physiologic GEF (*g*uanine nucleotide *e*xchange *f*actor) for Rab3A. Consistent with a role of Rab3A in synaptic vesicle exocytosis, GRAB regulates depolarization-induced release of dopamine from PC12 cells and nicotinic agonist-induced hGH release from bovine adrenal chromaffin cells. The association of InsP6K1 with GRAB fits with a role for InsP7 in vesicle exocytosis.

Introduction

Rab3A is a member of the Rab family of small G proteins that influence the trafficking of vesicular membranes in many types of cells. Rab3A is a neuronally specific protein localized to synaptic vesicles and involved in regulating neurotransmitter release (Oberhauser et al., 1992; Padfield et al., 1992; Holz et al., 1994; Johannes et al., 1994; Chung et al., 1999). Rab3A appears to limit the amount of neurotransmitter released in response to calcium entry. It does so by augmenting (in mice) the neurotransmitter release with targeted deletion of Rab3A (Geppert et al., 1997). Like other small G proteins, Rab3A cycles between the inactive GDP bound and active GTP bound forms. In the cytoplasm, Rab3A is maintained in its inactive GDP bound state by the Rab GDP-dissociation inhibitor (GDI) (Novick and Zerial, 1997; Geppert and Sudhof, 1998). When Rab3A binds to synaptic vesicles, GDI is displaced by a GDI displacement factor leading to exchange of GDP for GTP. Following vesicle fusion and hydrolysis of GTP by the intrinsic GTPase activity of Rab3A, GDI facilitates the movement of the GDP bound form of Rab3A back into the cytoplasm, thereby reinitiating the cycle. According to this model, the exchange of GTP and GDP on Rab3A is a crucial part of the protein functioning and regulating neurotransmitter release. For most small G proteins, this exchange is catalyzed by guanine nucleotide exchange factors (GEF). In *C. elegans*, a protein designated aex-3 displays GEF activity for Rab3A (Iwasaki et al., 1997), while Takai and associates have a mammalian homolog with GEF activity for Rab3A (Wada et al., 1997). Definitive evidence for a protein as the physiologic GEF for Rab3A has not heretofore been provided.

In efforts to clarify functions of inositol hexaphosphate kinase-1 (InsP6K1), an enzyme that synthesizes the inositol pyrophosphate diphosphoinositol-pentakisphosphate (InsP7) (Voglmaier et al., 1996; Saiardi et al., 1999), we conducted yeast two-hybrid analysis. In this investigation, we have identified a novel protein designated GRAB (*g*uanine nucleotide exchange factor for *Rab*3A). We show that GRAB displays GEF activity for Rab3A, a GTPase that physiologically regulates synaptic vesicle exocytosis (Geppert et al., 1997). GRAB is a physiologic GEF for Rab3A, as depletion of endogenous GRAB markedly reduces GTP loading of Rab3A. Binding of GRAB to Rab3A is mediated by a coiled-coil domain of GRAB, which also mediates its binding to InsP6K1 so that InsP6K1 and Rab3A compete for binding to GRAB. A role for GRAB in depolarization-induced release of dopamine from PC12 cells and hGH release from bovine adrenal chromaffin cells is indicated by augmentation of such release in cells depleted of endogenous GRAB and inhibition of such release in cells transfected with GRAB.

Results

InsP6K1 Binds GRAB through a Coiled-Coil Domain

We conducted yeast two-hybrid analysis utilizing full-length InsP6K1. One of the eight positive clones is GRAB. Utilizing various constructs of GRAB, we have localized the domain that binds InsP6K1 to a coiled-coil area comprising amino acids 70–160 (Figure 1). We demonstrate direct interactions between the proteins in that GST-GRAB beads pull down His-InsP6K1, while GST-14.3.3 beads, used as a negative control, fail to pull down InsP6K1 (Figure 1B). InsP6K1 and GRAB interact in intact cells, as they are pulled down together in HEK293 cells transfected with tagged forms of the two proteins (Figure 1C).

InsP6K1 and InsP6K2 display 70% sequence identity with the identity being greatest in the catalytic domain in the C-terminal portion, while there are substantial differences between the two proteins in the N-terminal portion (Saiardi et al., 1999). InsP6K2 fails to bind GRAB in yeast two-hybrid experiments (data not shown).

Screen of a lambda phage cDNA library of rat brain reveals a 1.1 kb open reading frame encoding a 378 amino acid sequence comprising the full length of GRAB (Figure 2A). The observed starting position of GRAB is

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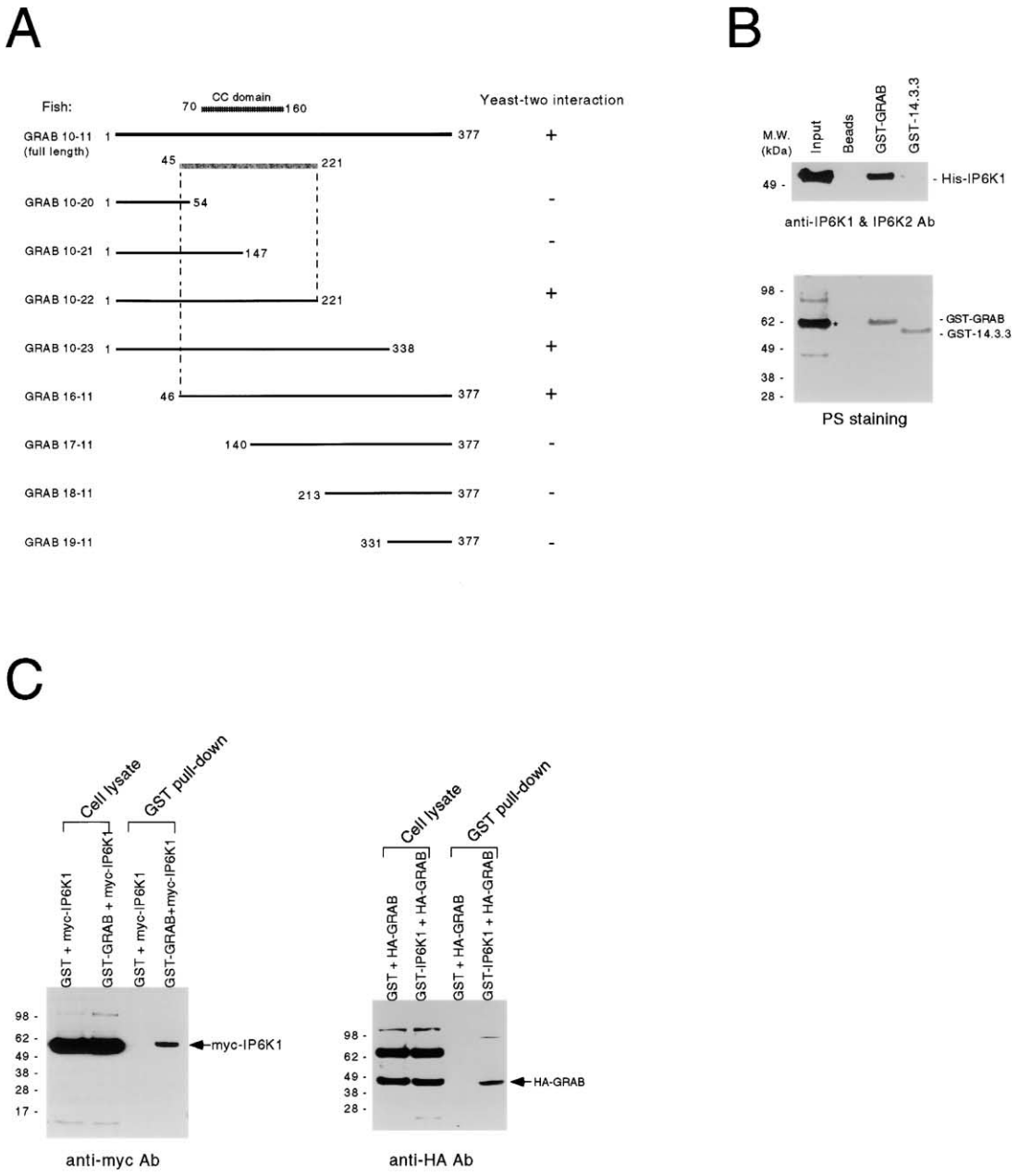

Figure 1. GRAB Interacts with InsP6K1 Both In Vitro and In Vivo

(A) The N-terminal part of GRAB containing the coiled-coil domain associates with InsP6K1 (InsP6K1) in the yeast two-hybrid system. Truncations of GRAB were constructed in pPC86 and cotransformed with pPC97-full-length InsP6K1 into PJ69 yeast cells. Constructs showing both histidine and adenine prototrophy as well as β-gal activity are listed as positive (+). Other constructs are listed as negative (−).

(B) In vitro binding of GRAB and InsP6K1. Assays were performed using His6-tagged and GST-tagged fusion proteins that were expressed in bacteria and purified as described. Indicated GST fusion proteins (final concentration, 10 μg/ml) were incubated with His-tagged InsP6K1 (final concentration, 5 μg/ml) in 300 μl lysis buffer containing 0.25 mg/ml BSA for 30 min at 37°C and followed by 2 hr at 4°C. Samples were precipitated with Glutathione Sepharose 4B beads and analyzed by Western blot using antibody against InsP6K1. Ponceau S (PS) staining of the membrane shows that the same amount of GST-GRAB and GST-14.3.3 (as a control) has been pulled down. BSA in the binding reaction was also stained and is represented by an astarisk. In the lane designated "Input," 10 μl unprecipitated protein sample was loaded.

(C) GRAB associates with InsP6K1 in cotransfected HEK293 cells. Indicated constructs were cotransfected into the cells using the calcium phosphate method. After 48 hr, cells were harvested and lysed in lysis buffer. Samples were pulled down with Glutathione Sepharose 4B beads and visualized by immunoblotting with anti-myc or anti-HA antibody.

likely correct as we have obtained the same initiating sequence in five separate phage preparations. Moreover, Western blot analysis of a brain lysate with antiserum to GRAB reveals a molecular weight of about 42 kDa, closely similar to that predicted from the amino acid sequence (Figure 2B). GRAB displays greater than 50% amino acid sequence similarity to Rabin3 (Brondyk et al., 1995), though their nucleotide sequences show only about 10% similarity. GRAB is 83 amino acids shorter than Rabin3 in the N-terminal portion, and hu-

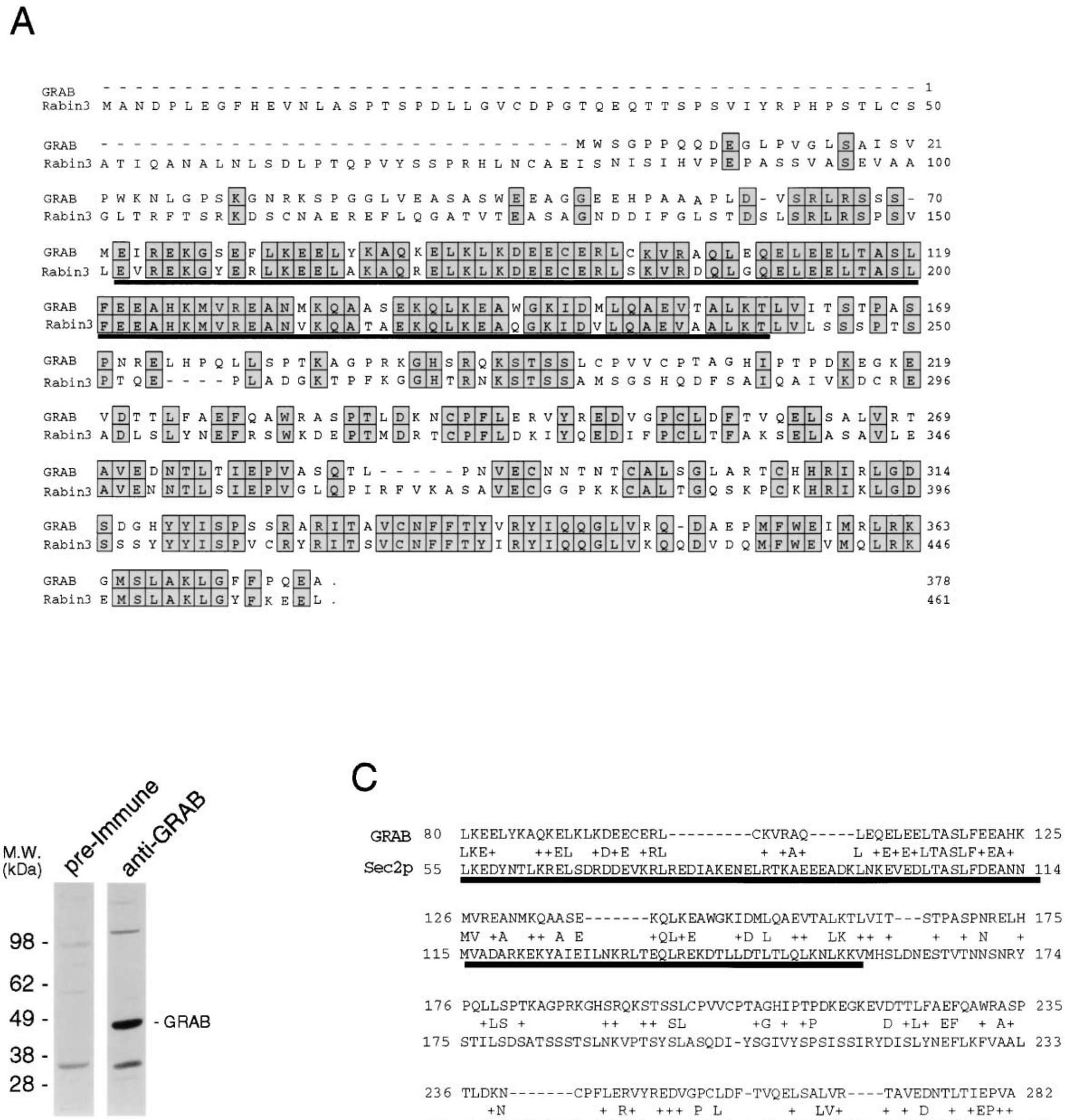

Figure 2. GRAB Is a Novel Protein Containing a Coiled-Coil Domain

(A) Amino acid sequence alignment of rat GRAB (GenBank accession number AY026049) and rat Rabin3 (GenBank accession number U19181). Sequences were aligned using DNAstar software. The portion of coiled-coil domain is demarcated by a solid line.

(B) Western blot of rat brain lysate proteins with preimmune or anti-GRAB serum. The position of GRAB protein is indicated.

(C) Amino acid sequence alignment of rat GRAB and yeast Sec2p (GenBank accession number X52147). Sequences were aligned using NCBI PSI-Blast program and the calculated e value is 6×10^{-4}. The portion of coiled-coil domain is demarcated by a solid line. The similarity between GRAB and Sec2 extends to both coiled-coil domain and other portions of the protein.

man genes for GRAB and Rabin3 are localized to chromosome 11 and 12, respectively. Thus, GRAB and Rabin3 are products of separate genes.

Database analysis indicates that amino acids 70–160 possess a coiled-coil pattern (Skehel and Wiley, 1998). A helical wheel projection of this region reveals selective clustering of hydrophobic amino acids in certain levels of the helix while hydrophilic amino acids cluster in other levels (data not shown). This pattern resembles that of most coiled-coil proteins and is important for their ability to interact with other proteins containing similar domains.

Analysis of databases for yeast reveals the highest homology of GRAB with the yeast protein Sec2 (Nair et al., 1990) (Figure 2C). The homology is substantial in the coiled-coil domain but also occurs in other parts of the protein. Sec2 is a GEF protein for Sec4 (Walch-Solimena et al., 1997).

Binding Interactions of GRAB and Rab3A

GRAB displays considerable homology to Sec2p, which is a GEF for Sec4p, a major yeast GTPase involved in vesicular secretion (Salminen and Novick, 1987). Various members of the Rab family, such as Rab3 and Rab8, are

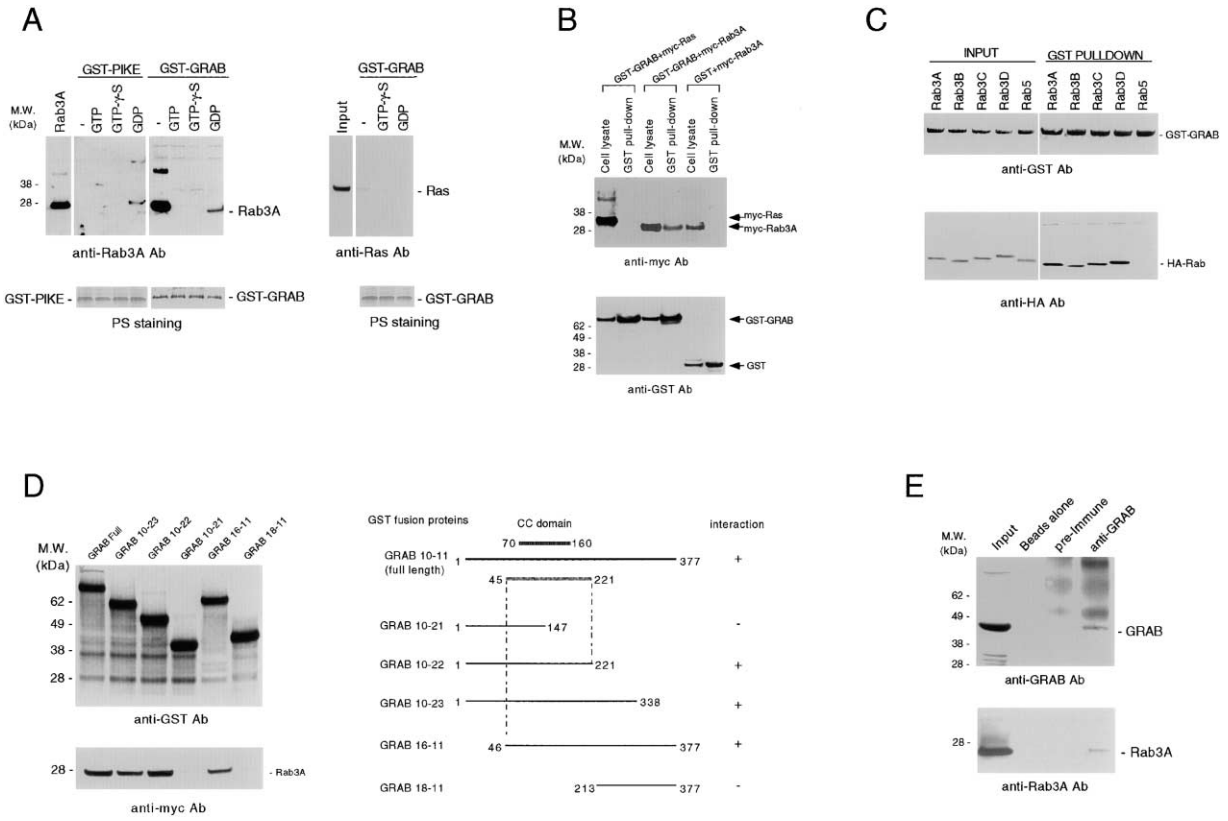

Figure 3. GRAB and Rab3A Associate In Vitro and In Vivo

(A) In vitro binding of GRAB and Rab3A. Assays were performed using purified recombinant Rab3A and GST-fusion proteins expressed in bacteria. GST-PIKE (as a control) or GST-GRAB protein (final concentration, 10 μg/ml) were incubated with recombinant Rab3A (final concentration, 15 μg/ml) in 300 μl lysis buffer containing 0.2 mg/ml BSA for 30 min at 37°C followed by 2 hr at 4°C. The incubation was performed in the presence or absence of guanine nucleotide, as indicated. Samples were precipitated with Glutathione Sepharose 4B beads and analyzed by Western blot using anti-Rab3A antibody. PS staining shows that the same amount of GST-GRAB and GST-PIKE had been pulled down. GST-GRAB was also incubated with Ras protein, but no interaction was detected, as shown on the right. In the lane "Input," 10 μl unprecipitated protein sample was loaded.

(B) GRAB associates with Rab3A in cotransfected HEK293 cells. Indicated constructs were cotransfected into cells using the calcium phosphate method. Samples were prepared as previously described, pulled down with Glutathione Sepharose 4B beads, and visualized by immunoblotting with anti-myc or anti-GST antibody.

(C) GRAB binds Rab3, but not Rab5 in cotransfected HEK293 cells. Indicated HA-tagged Rab constructs were cotransfected into cells with a GST-GRAB construct. Samples were pulled down with Glutathione Sepharose 4B beads and visualized by immunoblotting with anti-HA and anti-GST antibody. Cell lysate (0.3 ml) was used for GST pull-down assay, and 20 μl was loaded as Input.

(D) The coiled-coil domain of GRAB binds Rab3A. Truncations of GRAB were expressed as GST fusion proteins in transfected HEK293 cells. GRAB constructs were cotransfected into cells with myc-Rab3A construct. Samples were pulled down with Glutathione Sepharose 4B beads and visualized by immunoblotting with anti-GST or anti-myc antibody.

(E) Coimmunoprecipitation of GRAB and Rab3A from rat brain lysate. Rat brain lysate was immunoprecipitated with GRAB antiserum or preimmune serum that have been covalently linked to Protein G beads. The precipitates were blotted with rabbit polyclonal anti-GRAB as well as mouse monoclonal anti-Rab3A antibodies. Lane 1 contains an aliquot (5% of 200 μl total input) of the brain lysate used for coimmunoprecipitation.

mammalian orthologs of Sec4p. Since GRAB resembles Rabin3, which binds Rab3A, we explored binding between GRAB and Rab3A (Figure 3). Utilizing bacterially expressed GST-GRAB and Rab3A, we demonstrate binding between these proteins (Figure 3A). Selectivity is evident in the failure of GST-PIKE (Ye et al., 2000) to bind Rab3A. If GRAB were a GEF, one might expect its interactions with the GTPase Rab3A to be influenced by GTP or GDP. Indeed, GTP and GTP-γ-S markedly inhibit the binding of GRAB and Rab3A. GDP also exerts some inhibition, though substantially less than GTP. Evidence for the selectivity of the GRAB-Rab3A interaction also comes from experiments showing that GRAB fails to bind to Ras in the presence or absence of GTP-γ-S and GDP (Figure 3A).

To examine interactions of GRAB and Rab3A in intact cells, we transfected HEK293 cells with tagged forms of GRAB and Rab3A (Figure 3B). We observe robust interactions of the two proteins. To examine the selectivity of these interactions, we utilized HEK293 cells transfected with Rab3A, Rab3B, Rab3C, Rab3D, and Rab5 (Figure 3C). Rab3B, Rab3C, and Rab3D bind GRAB to a similar extent, as does Rab3A. By contrast, Rab5 fails to bind GRAB.

To ascertain the domain of GRAB that interacts with Rab3A, we transfected HEK293 cells with Rab3A as well

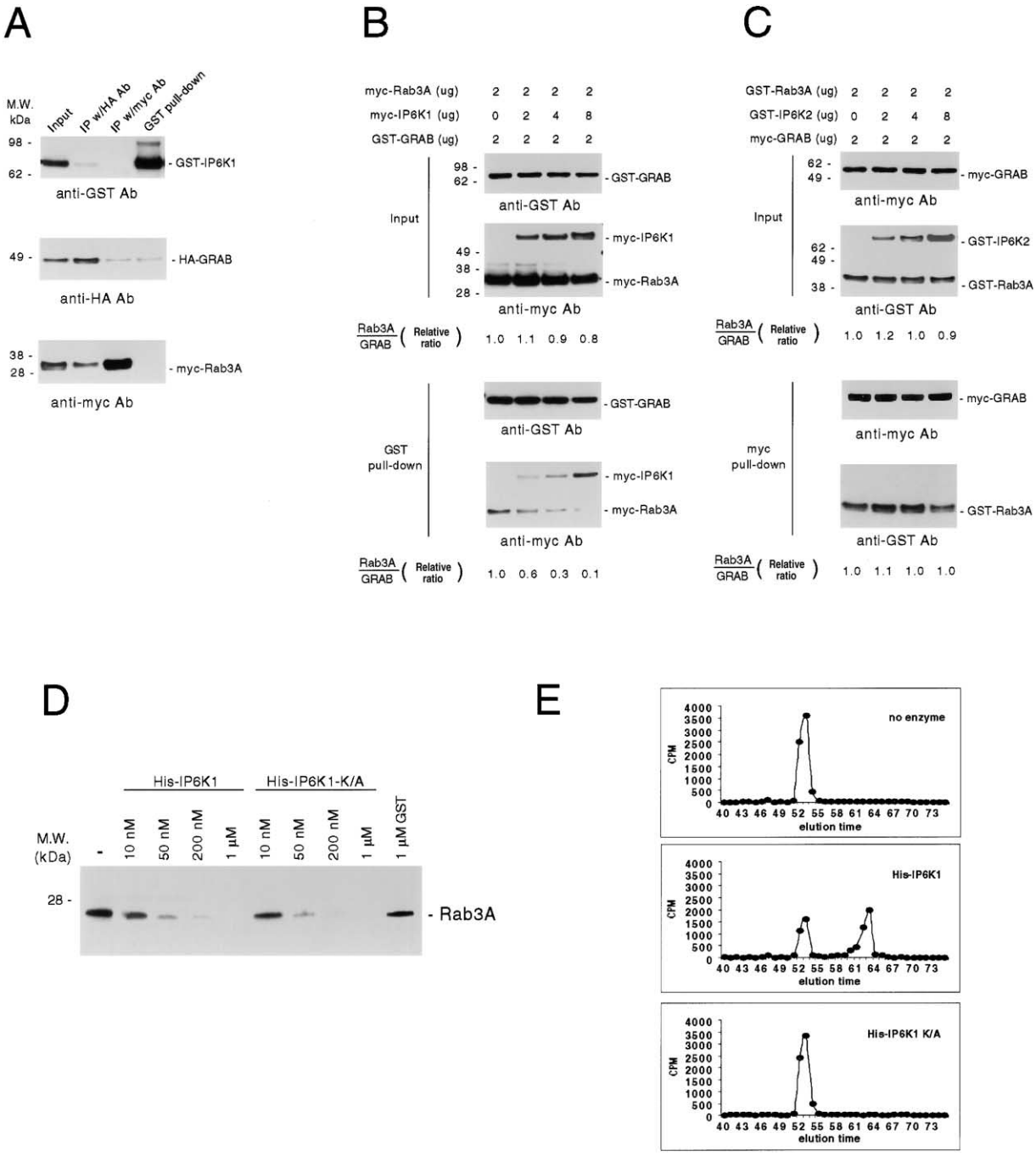

Figure 4. InsP6K1 Interrupts Rab3A-GRAB Interaction

(A) InsP6K1 does not directly interact with Rab3A. Constructs of GST-InsP6K1, HA-GRAB, and myc-Rab3A were cotransfected into HEK293 cells using the calcium phosphate method. Samples were prepared as previously described and pulled down with Glutathione Sepharose 4B beads, with anti-HA antibody, or with anti-myc antibody. The precipitates were visualized by immunoblotting with anti-GST, anti-HA, or anti-myc antibodies as indicated. Cell lysate (0.3 ml) was used for GST pull-down assay, and 20 µl was loaded as Input.

(B) InsP6K1 interrupts Rab3A-GRAB interaction in cotransfected HEK293 cells. Constructs of GST-GRAB, myc-InsP6K1, and myc-Rab3A were cotransfected into cells using the indicated amount. Samples were pulled-down with Glutathione Sepharose 4B beads. The precipitates were visualized by immunoblotting with anti-GST and anti-myc antibodies as indicated.

(C) InsP6K2 does not interrupt Rab3A-GRAB interaction. Constructs for myc-GRAB, GST-InsP6K2, and GST-Rab3A were cotransfected into HEK293 cells using the indicated amount. Samples were immunoprecipitated with anti-myc antibody and protein A/G beads. The precipitates were visualized by immunoblotting with anti-GST or anti-myc antibody as indicated.

(D) InsP6K1 interrupts Rab3A-GRAB interaction in rat brain lysate. Rat brain lysate was immunoprecipitated with anti-GRAB antiserum in the presence of indicated amounts of purified recombinant proteins expressed in bacteria. The precipitates were blotted with mouse monoclonal anti-Rab3A antibodies. His-InsP6K1-K/A is a catalytically inactive mutant InsP6 kinase.

(E) InsP6 kinase activities of recombinant His-InsP6K1 and His-InsP6K1-K/A. Assays were performed in 10 µl of reaction mixture containing 10 ng recombinant InsP6K1, 5 µM InsP6, and 40 nM [^{3}H]InsP6. Samples were incubated at 37°C for 2 hr. InsP6 (the first peak) was separated from InsP7 (the second peak) by HPLC.

as with the various fragments of GRAB whose binding to InsP6K1 had been evaluated in yeast two-hybrid analysis (Figure 3D). The fragments of GRAB that interact with Rab3A are the same as those that interact with InsP6K1, indicating that the coiled-coil domain of GRAB, amino acids 70–160, is primarily responsible for interactions with Rab3A.

To determine whether GRAB and Rab3A are associated in intact brain, we immunoprecipitated with antibodies to GRAB (Figure 3E). We observe selective coimmunoprecipitation of GRAB and Rab3A, establishing that they interact in intact brain. We are unable to quantify the proportion of endogenous levels of these proteins that coprecipitate.

Our finding that the binding of GRAB to InsP6K1 and Rab3A involves the same domain of GRAB suggests that InsP6K1 and Rab3A may compete for binding to GRAB. First, we examined the possibility that InsP6K1 might itself interact directly with Rab3A (Figure 4A). HEK293 cells were cotransfected with InsP6K1, GRAB, and Rab3A with different tags. Pull-down experiments confirm that GRAB interacts with InsP6K1 and with Rab3A. However, we observe no binding of InsP6K1 to Rab3A. It is difficult to quantify the extent of coprecipitation of InsP6K1 and GRAB, as we don't know whether the two proteins are expressed to a similar extent. In experiments involving the 3 proteins, GRAB, InsP6k1, and Rab3A, binding of InsP6k1 to GRAB may be reduced by competition with Rab3A.

To determine whether InsP6K1 influences the GRAB-Rab3A interaction, we cotransfected the HEK293 cells with tagged forms of Rab3A and GRAB in the presence of increasing amounts of InsP6K1 (Figure 4B). InsP6K1 inhibits the GRAB-Rab3A interaction in a concentration-dependent fashion.

We wondered whether the ability of InsP6K1 to disrupt the binding of GRAB to Rab3A is a function of the InsP6K1 protein or of the InsP7 that it synthesizes. As InsP6K2 does not bind to GRAB, we compared the ability of InsP6K1 and InsP6K2, which possess similar capacities to form InsP7 (Saiardi et al., 1999), to influence the GRAB-Rab3A interaction (Figure 4C). While InsP6K1 robustly blocks the binding of GRAB to Rab3A, InsP6K2 is without effect. Thus, it is the InsP6K1 protein itself which regulates interaction between Rab3A and GRAB.

To test whether InsP6K1 disrupts the GRAB-Rab3A interaction in intact brain, we coimmunoprecipitated Rab3A with antibodies to GRAB in the presence of different concentrations of InsP6K1 (Figure 4D). Both wild-type and catalytically inactive mutant InsP6K1 inhibit the binding of endogenous GRAB and Rab3A with 50% inhibition at 10–50 nM InsP6k1. The catalytically inactive InsP6K1 (InsP6K1-K/A) contains a mutation (lysine to alanine) in the catalytic domain (Saiardi et al., 1999) that disrupts kinase activity as confirmed by catalytic assay (Figure 4E). Comparable influences of wild-type and mutant InsP6K1 on the GRAB-Rab3A interaction indicate that the kinase activity is not required for this regulation.

Endogenous GRAB Is a GEF for Rab3A and Regulates Depolarization-Induced Vesicular Release Processes

The structural similarity of GRAB to Sec2, which is a GEF in yeast, suggested that GRAB might be a GEF for the GTPase Rab3A. GEF activity causes dissociation of GDP from GTPases and augments the binding of GTP. We monitored the ability of bacterially expressed GRAB to influence the dissociation of [^{3}H]GDP from purified bacterially expressed Rab3A (Figure 5A). GRAB substantially enhances the rate of dissociation of GDP from Rab3A. We also monitored the influence of GRAB on the binding of [^{35}S]GTP-γ-S to Rab3A (Figure 5B). GRAB increases the steady-state binding of GTP to Rab3A about two-fold. The GDP-GTP exchange activity of GRAB on Rab3A is greater than that of some GEF proteins, such as CalGEFII on Rap1 and Mss1 on Rab3 (Kawasaki et al., 1998; Yamashita et al., 2000), but slightly lower than effects of Rab3GEP upon Rab3A (Iwasaki et al., 1997). Since we used bacterially expressed Rab3A and GRAB proteins, the observed GEF activity may be an underestimate.

To ascertain whether GRAB is a physiologic GEF for

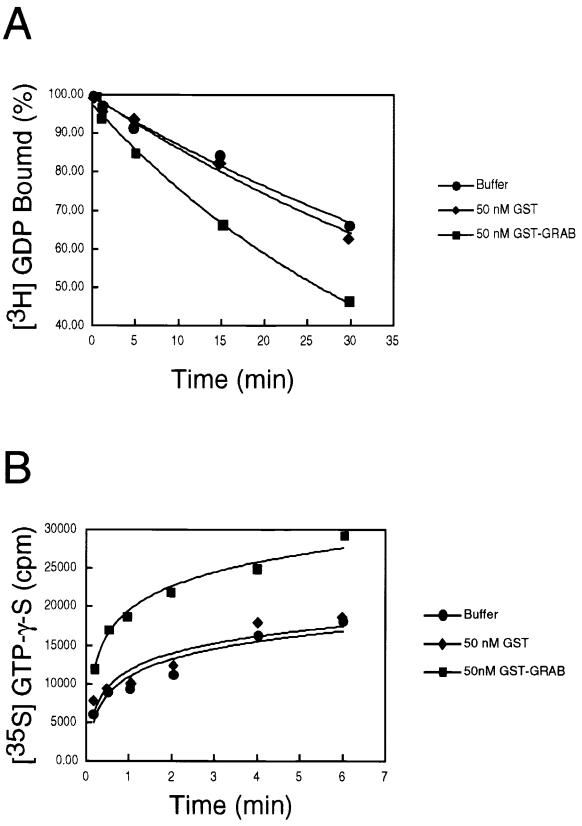

Figure 5. GRAB Stimulates Guanine Nucleotide Exchange on Rab3A

(A) GRAB accelerates GDP release from Rab3A. Rab3A (50 nM) was preloaded with 0.5 μM [^{3}H]GDP for 30 min at 30°C. Purified GST-GRAB (50 nM), GST protein (50 nM), or control buffer was added together with 0.5 mM cold GTP at the start of the assay. At the time intervals indicated, the Rab3A bound radioactivity was measured by a filter binding assay. Stimulation of GDP dissociation is expressed as the percentage of [^{3}H]GDP bound to Rab3A before the addition of cold ATP.

(B) GRAB stimulates GTP binding to Rab3A. Rab3A (50 nM) was incubated with 50 nM GST-GRAB, GST protein, or control buffer for 30 min at 37°C. [^{35}S]GTP-γ-S (2 μCi/ml) (3 μM) was added to start the assay. At the indicated time points, [^{35}S]GTP-γ-S bound to Rab3A was measured by a filter binding assay

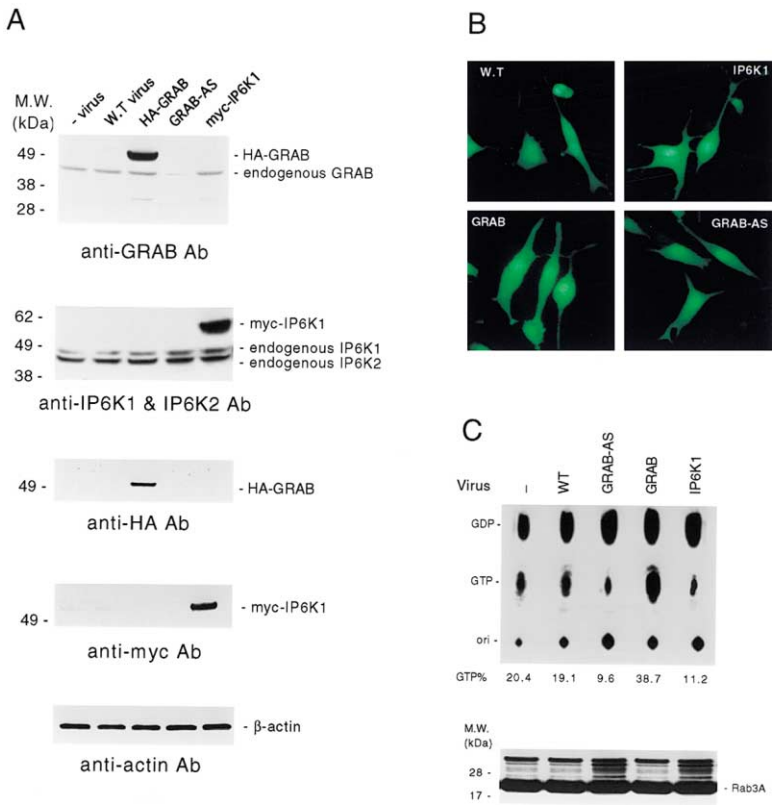

Figure 6. GRAB Acts as a Physiologic Guanine Nucleotide Exchange Factor for Rab3A in PC12 Cells

(A) Generation of adenovirus. After homologous recombination, screening, amplification, and purification, high-titer adenoviruses were generated. PC12 cells were infected with indicated virus for 20 hr, and cell lysate was analyzed by immunoblotting with indicated antibodies. Abbreviations: − virus, uninfected cells; W.T. virus, cells infected with virus containing no foreign gene; HA-GRAB, virus containing HA-tagged GRAB gene; GRAB-AS, virus containing antisense construct against GRAB cDNA; myc-InsP6K1, virus containing myc-tagged InsP6K1 gene. Antibodies against GRAB and InsP6K1 detected both recombinant and endogenous proteins. Anti-actin antibody was used to determine if the same amount of cell lysate was loaded in each lane.

(B) Growth and morphology of PC12 cells are not altered after infection with indicated virus. In the viral system we used, there is a GFP reporter gene incorporated into the viral backbone, permitting monitoring of adenoviral infection by GFP expression. In our experiment, viruses were used at moi values of 100 and 75%–80% infection efficiency was obtained routinely.

(C) GRAB enhances GTP loading on Rab3A. PC12 cells were differentiated for 7 days with NGF and then infected with indicated viruses for 20 hr. Metabolic labeling was performed in phosphate-free DMEM with 0.5 mCi/ml $[^{32}P]H_3PO_4$ for 4 hr at 37°C. Rab3A was immunoprecipated with polyclonal anti-Rab3A antibody, and bound guanine nucleotides were eluted and separated on TLC plates. The percent of GTP was calculated as cpm in GTP/(cpm in GTP + cpm in GDP). Western blot with mouse monoclonal anti-Rab3A antibody shows that the same amount of Rab3A was precipitated from each reaction.

Rab3A in intact cells, we utilized PC12 cells in which an adenovirus was utilized to infect the cells with a variety of proteins (Figure 6). We observe robust expression of infected GRAB and InsP6K1 (Figure 6A). Infection with antisense to GRAB leads to depletion of endogenous GRAB. Viral treatment of cells sometimes can be toxic leading to morphologic changes, especially with a very high level of infection. Accordingly, we employed concentrations of the virus leading to infection of about 75% of the cells. Under these conditions, the morphology of cells infected with InsP6K1, GRAB, and antisense to GRAB does not differ from wild-type cells (Figure 6B).

We monitored GEF activity in intact PC12 cells infected by adenovirus with InsP6K1, GRAB, and antisense to GRAB. Endogenous guanine nucleotides were labeled by incubating the cells with ^{32}P-orthophosphate (Figure 6C). Levels of radiolabeled GTP and GDP are the same in cells not infected with virus or infected with wild-type virus. Infection with antisense to GRAB reduces the GTP loading of Rab3A by 50%. This establishes that endogenous GRAB serves as a physiologic GEF for Rab3A. Infection with GRAB doubles GTP loading of Rab3A compared to levels in wild-type cells. Infection with InsP6K1 reduces GTP loading of Rab3A by 50% compared to wild-type samples, consistent with its competition with Rab3A for binding to GRAB.

Rab3A is well established to regulate exocytic release of neurotransmitters from neuronal tissue and human growth hormone (hGH) from adrenal chromaffin cells. Rab3A is a negative regulator of the release process, as mice with targeted deletion of Rab3A manifest augmented release (Geppert et al., 1997), and antisense constructs to Rab3A increase release (Johannes et al., 1994) as do peptides that compete with Rab3A (Oberhauser et al., 1992; Padfield et al., 1992). Moreover, overexpression of Rab3A inhibits release processes (Chung et al., 1999; Holz et al., 1994). GEF proteins augment the activity of their associated GTPases. Accordingly, if GRAB is a physiologic GEF for Rab3A, then GRAB should be a negative regulator of release. We examined this possibility by monitoring depolarization-induced release of dopamine from PC12 cells and of human growth hormone (hGH) from bovine adrenal chromaffin cells (Figure 7). We utilized PC12 cells with adenoviral infection leading to expression of GRAB, antisense to GRAB, and InsP6K1 just as was done for the experiments monitoring GTP loading of Rab3A in these cells. Endogenous stores of dopamine were labeled via the physiologic dopamine transporter utilizing $[^3H]$dopamine (Sandberg et al., 1989; Steiner et al., 1996). Under these conditions, it has previously been shown that potassium depolarization-induced release of $[^3H]$dopamine is a faithful representation of the release of endogenous catecholamines (Levi et al., 1988). In our experiments, potassium depolarization leads to a tripling of basal release of $[^3H]$dopamine, with the depolarization-induced release being abolished by the calcium chelator EGTA (Figure 7A, data not shown). Infection with wild-type

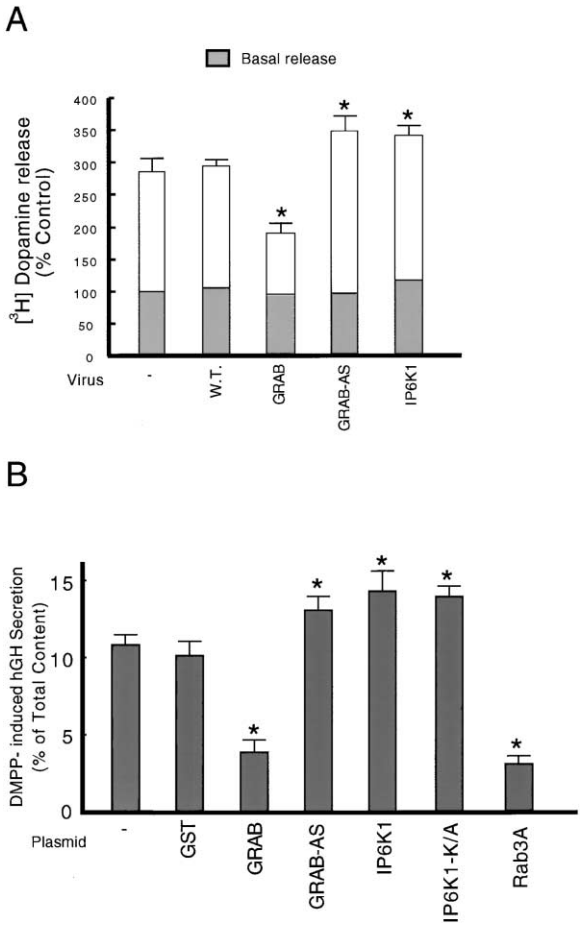

Figure 7. GRAB Regulates Mediator Release from PC12 Cells as Well as from Bovine Adrenal Chromaffin Cells

(A) GRAB regulates dopamine release from PC12 cells. PC12 cells were differentiated for 7 days with NGF and then infected with indicated viruses for 20 hr. After incubation with [^{3}H]dopamine for 60 min, release was triggered by addition of 40 mM KCl for 2 min at 37°C. Transmitter released in the absence and presence of KCl is indicated by gray and white bars respectively. The amount of transmitter released in the absence of KCl from the uninfected cells is designated 100% of control. GRAB-reduced and GRAB-AS- and InsP6K1-increased dopamine release compared to wild-type ($p < 0.01$, Student's t test). The results are the means of three independent experiments. Bars indicate mean ± SEM. Asterisk indicates statistically significant.

(B) GRAB regulates nicotinic agonist-induced hGH release from bovine adrenal chromaffin cells. Cultured primary bovine adrenal chromaffin cells were cotransfected with indicated plasmids and the expression vector pXGH containing hGH cDNA. Six days after transfection, hGH release was evaluated by stimulating cells with 20 μM 1,1-dimethyl-4-phenylpiperazinim (DMPP, a nicotinic agonist) at 37°C for 5 min. hGH released into the medium and retained in the cells was measured using a radioimmunoassay for hGH. Secretion is expressed as the percentage of total hGH (hGH released into the medium and hGH retained in the cells). hGH release in the absence of DMPP was 2%–3%, and overexpression of recombinant proteins did not affect basal secretion. GRAB- and Rab3A-reduced and GRAB-AS-, InsP6K1-, and InsP6K1-K/A-increased DMPP-evoked hGH release compared to wild-type ($p < 0.01$, Student's t test). The results are the mean of three independent experiments. Bars indicated mean ± SEM.

virus does not alter basal or depolarization induced release, ensuring that viral infection does not damage the release process. Infection with antisense to GRAB significantly augments depolarization-induced release of dopamine, establishing that endogenous GRAB physiologically regulates release. By contrast, infection with GRAB reduces by 50% depolarization-induced release. Infection with InsP6K1 significantly augments release, presumably reflecting a competition with Rab3A for binding to GRAB. None of the infection conditions alter basal release, implying that the infections selectively influence processes associated with depolarization-induced transmitter release, presumably the vesicular exocytic processes regulated by Rab3A and GRAB. The similar effects of GRAB antisense and InsP6K1 on dopamine release and the opposite effect of GRAB infection closely parallel the relative influences of these proteins on GTP loading of Rab3A in the same cells. This finding indicates that the physiologic regulation by endogenous GRAB of transmitter release reflects its GEF activity on Rab3A.

We obtained additional evidence for a role of GRAB in vesicular release using bovine adrenal chromaffin cells in which we monitored the release of transfected hGH and the effects of cotransfected GRAB, antisense to GRAB, Rab3A, and InsP6K1. We elicited hGH release by stimulating nicotinic cholinergic receptors with the nicotinic agonist DMPP (Holz et al., 1995; Wick et al., 1993) (Figure 7B). In confirmation of the work of others (Holz et al., 1994; Johannes et al., 1994), we show that Rab3A markedly reduces hGH release. Antisense to GRAB significantly augments depolarization-induced hGH release, further establishing that in this system, as in the PC12 cells, depolarization-induced release is regulated by endogenous GRAB. Transfection with GRAB reduces hGH release to a similar extent as Rab3A, consistent with coordinated actions of GRAB and Rab3A in negatively regulating the release process. InsP6K1 significantly increases hGH release, consistent with its competition with Rab3A for binding to GRAB. We also find that InsP6K1-K/A, a catalytically inactive InsP6K1, exerts a similar effect as catalytically active InsP6K1. Therefore, it is InsP6K1 protein (rather than InsP7) that regulates growth hormone secretion.

Localizations and Ontogeny of GRAB

Northern blot analysis indicates a widespread distribution of mRNA for GRAB (Figure 8A). Substantial levels of a 2.4 kb GRAB mRNA are evident in brain, spleen, heart, lung, liver, skeletal muscle, kidney, and testes. Testis also displays a larger mRNA species, about 3.4 kb.

In contrast to the widespread distribution of GRAB mRNA, GRAB protein, analyzed by Western blot, is fairly selectively localized to brain (Figure 8B). We do not know why some peripheral tissues displaying GRAB mRNA fail to express GRAB protein. In testes, many transcribed genes are often not translated to protein. In other tissues, variations in translation as well as protein turnover could account for less GRAB protein. A 42 kDa protein band is robustly expressed in cerebellum and cerebral cortex with somewhat lower levels in the hippocampus. In the cerebral cortex, we also observe a larger molecular weight band at about 100 kDa and an even fainter

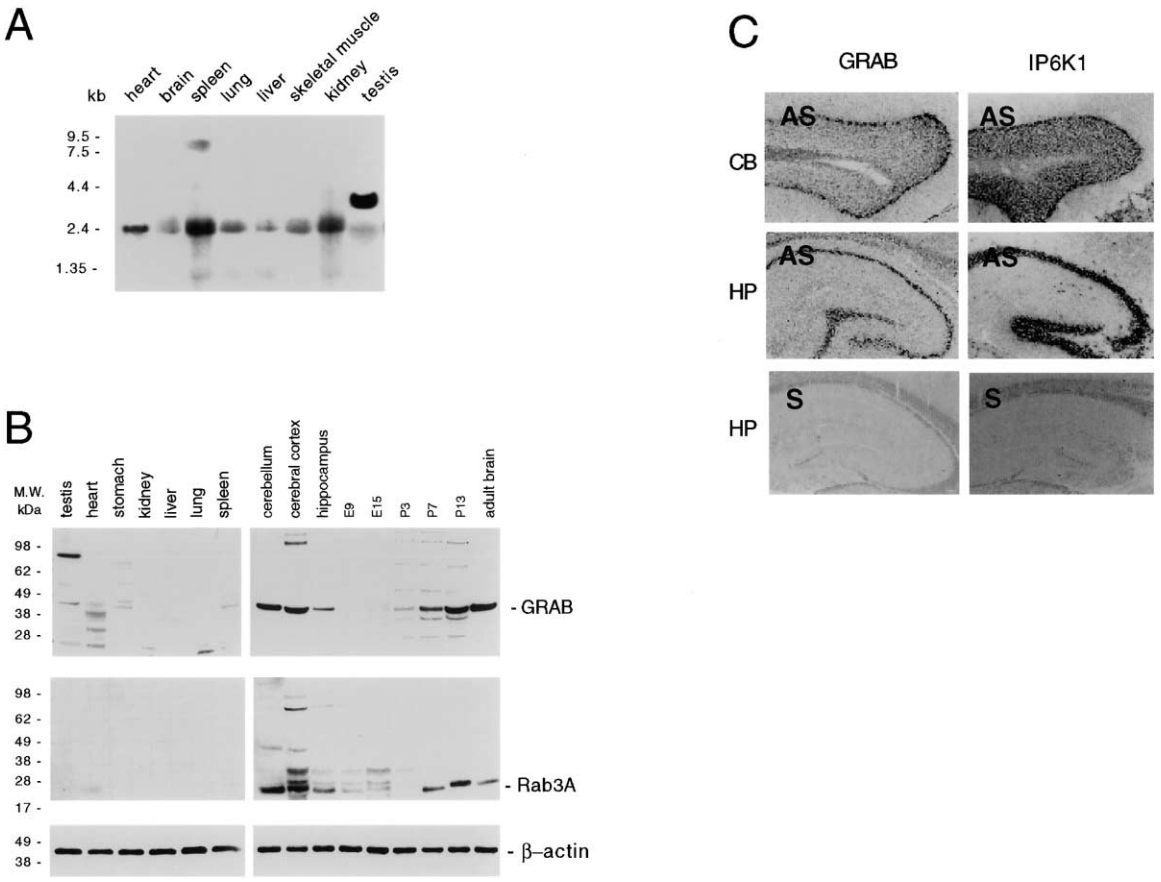

Figure 8. Tissue Distribution of GRAB

(A) Rat multiple tissue Northern blot was hybridized with a probe corresponding to full-length GRAB. Size markers are indicated.

(B) Western blotting of protein lysate from various adult rat tissues and rat brain at various developmental stages. Immunoblotting was performed using a protein A beads purified rabbit polyclonal antibody against GRAB, mouse monoclonal antibody against Rab3A, and anti-actin antibody, respectively. P, postnatal day; E, embryonic day.

(C) GRAB and InsP6K1 colocalize in brain. In situ hybridization of adult rat hippocampus (HP) and cerebellum (CB) shows similar tissue distributions for the two proteins. AS, antisense probe; S, sense probe. Sense control probes used at equal concentration generated no specific signal.

band at 120 kDa. We observe a faint 42 kDa band in the spleen but negligible levels of immunoreactivity at this molecular weight in lung, liver, kidney, and stomach. The heart displays a relatively faint band at 42 kDa and somewhat more prominent bands at lower molecular weights. The testis displays a 42 kDa band and a more prominent band at about 80 kDa.

GRAB is developmentally expressed (Figure 8B). We observe negligible levels at embryonic stages E9 and E15, while GRAB levels increase progressively at postnatal ages P3, P7, and P13 with maximal levels in adult brain.

If GRAB functions physiologically in concert with Rab3A, the two proteins ought to display similar localizations. In confirmation of previous studies (Mizoguchi et al., 1989), we find Rab3A expression largely restricted to the brain with high levels in the cerebellum, cerebral cortex, and hippocampus. Like GRAB, Rab3A is developmentally regulated. Very low levels are observed at E9 and E15, with low levels at P3 increasing at P7 and P13 (Figure 8B).

To observe cellular localizations, we conducted in situ hybridization for GRAB mRNA (Figure 8C). We compared localizations of GRAB with those of InsP6K1. In the cerebellum, highest expression of both GRAB and InsP6K1 occurs in Purkinje cells. The granule cell layer contains substantial levels of InsP6K1 but very little GRAB. In the hippocampus both GRAB and Rab3A are concentrated in the pyramidal cell layer of CA1, 2, and 3, as well as the dentate gyrus. InsP6K1 is enriched more in the hippocampus than GRAB.

Discussion

In the present study, we have identified GRAB as a novel mammalian GEF for Rab3A. GRAB appears to be the mammalian ortholog of the yeast GEF Sec2p, as Sec2p is the yeast protein displaying greatest homology to GRAB, and both proteins have GEF functions. Sec2 is the physiologic GEF for Sec4p, a small GTPase of the Rab family and thought to be the yeast ortholog of Rab3A. In confirmation of the homology of GRAB and yeast Sec2p, we provide compelling evidence that GRAB is the physiologic GEF for Rab3A. GRAB binds

robustly to Rab3A. Antisense to GRAB, which depletes endogenous levels of GRAB in PC12 cells, markedly diminishes the GTP loading of Rab3A and augments depolarization-induced transmitter release from PC12 cells and of hGH from adrenal chromaffin cells.

Other proteins display GEF activity for Rab3A. De-Camilli and associates (Burton et al., 1993) as well as Novick and associates (Moya et al., 1993) studied yeast with a mutant sec4 that manifested abnormalities in growth and vesicular disposition and cloned from a mammalian cDNA library a protein, Mss4, which rescued these abnormalities and displayed GEF activity toward Sec4p. Nuoffer et al. (1997) obtained evidence that Mss4 does not function as a GEF, but instead interacts with the transient guanine nucleotide-free state of Sec4p. Takai and collaborators (Wada et al., 1997) purified from rat brain a protein displaying GEF activity toward Rab3A, which they designated Rab3GEP. Mss4 acts on a wide range of Rab family members, whereas Rab3GEP is selective for members of the Rab3 family, a specificity similar to that of GRAB. Brondyk et al. (1995) identified Rabin3 in a yeast two-hybrid screen as a protein that binds to Rab3A. Rabin3 displays homology to Sec2p, a yeast GEF. However, Rabin3 fails to display GEF activity for Rab3A.

GEF proteins tend not to be promiscuous, but display varying degrees of selectivity for GTPases with which they are associated (Feig, 1994). Our finding that depletion of endogenous GRAB reduces GTP loading of Rab3A and establishes GRAB as a physiologic regulator of Rab3A's GTPase activity. Evidence for a physiologic influence of Mss4 or Rab3GEP on any member of the Rab3 family has not been published. However, it is conceivable that Rab3A's GTPase activity might be influenced by more than a single GEF so that GEF proteins (in addition to GRAB) may physiologically regulate Rab3A.

Rab3A is the principal small GTPase that regulates synaptic vesicle exocytosis (Lin and Scheller, 2000; Novick and Zerial, 1997; Schimmoller et al., 1998). Our finding that disruption of GRAB alters transmitter release and Rab3A function in parallel indicates that GRAB also regulates exocytic events, presumably via Rab3A. Manipulation of GRAB may provide a tool to elucidate such events.

At what point in the cycling of synaptic vesicles might GRAB exert its GEF activity upon Rab3A? In its GTP bound state, Rab3A associates with the plasma membrane through its geranylgeranyl groups. Following hydrolysis of GTP, the GDP-Rab3A is extracted from the membrane in a complex with GDI, a cytosolic protein (Geppert and Sudhof, 1998). Rab3A is then recycled into a newly formed vesicle, perhaps through a secondary factor termed GDI dissociation factor (GDF) that displaces GDI. GEF activity is presumed to act upon Rab3A following association with a newly formed vesicle, serving to activate Rab3A by loading it with GTP, consistent with evidence that Rab3A is loaded with GTP after membrane binding (Chou and Jahn, 2000).

We identified GRAB as a protein that binds InsP6K1. InsP6K1 and InsP6K2 are the principal biosynthetic enzymes forming the inositol pyrophosphate InsP7. The exact biochemical functions of the inositol pyrophosphates have not been established. They have been suggested to serve as phosphate donors for proteins (Voglmaier et al., 1996). A role for InsP6 and InsP7 in vesicular physiology stems from observations that these inositol polyphosphates bind to clathrin-associated proteins that are involved in vesicle turnover (Voglmaier et al., 1992; Norris et al., 1995; Ye et al., 1995; Mizutani et al., 1997). Moreover, we have observed that targeted deletion of InsP6K in yeast leads to substantial disruptions of normal vacuole morphology (Saiardi, et al., 2000) due to an altered trafficking of vesicular membranes during endocytosis (A.S., C. Sciambi, C. Colantuoni, J. Pevsner, B. Wendland, S.H.S., unpublished).

In the present study, we have observed that both InsP6K1 and Rab3A bind competitively to the coiled-coil domain of GRAB and hence compete in regulating the GTP loading of Rab3A as well as the depolarization-induced release of dopamine from PC12 cells and hGH from adrenal chromaffin cells. InsP6K1, which binds GRAB, disrupts the binding of GRAB to Rab3A, whereas InsP6K2, which does not bind GRAB, fails to disrupt the binding of GRAB to Rab3A. Hence, the InsP6K1 protein, rather than the InsP7 that it synthesizes, influences interactions between Rab3A and GRAB.

Experimental Procedures

Materials

NGF, X-Gal, and IPTG were from Boehringer Mannheim (Indianapolis, IN). Molecular cloning reagents were purchased from New England Biolabs (Beverly, MA) or Promega (Madison, WI). Glutathione Sepharose 4B was from Pharmacia. Protein A/G-agarose was from Calbiochem (La Jolla, CA). All other reagents were purchased from Sigma (St. Louis, MO), except as indicated.

Yeast Two-Hybrid Screening and cDNA Cloning

Two-hybrid screening was conducted using the PJ69 yeast strain containing HIS3, ADE, and β-galactosidase (β-gal) reporter genes. Full-length InsP6k1 ORF was subcloned into SalI and NotI sites of yeast expression vector pPC97, encoding the GAL4 DNA binding domain. A rat hippocampal cDNA library was cloned into pPC86, containing the GAL4 transactivation domain. A total of 1.2×10^7 independent clones were screened, and eight positive clones were identified. A 1.0 kb cDNA fragment was isolated and characterized as a portion of the GRAB gene. The full-length GRAB cDNA was obtained by screening an adult rat brain cDNA library in λZAPII vector (Stratagene; La Jolla, CA). After screening a total of 1×10^6 clones, a full-length 1.1 kb open reading frame was obtained from seven overlapping inserts.

Expression of Fusion Protein in Bacteria and Production of Polyclonal Antisera

GRAB cDNA was subcloned in-frame with the N-terminal His6 tag of bacteria expression vector pET-28-c (Novagen; Madison, WI) or the N-terminal GST tag of bacteria expression vector pGEX-4T-2 (Pharmacia). The fusion proteins were overexpressed in BL21 *E. coli* cells grown in LB amp+ medium by induction with 1 mM IPTG for 3 hr after the culture had obtained an $OD_{600} = 0.5$. Protein purification was then performed using BugBuster His-Bind Purification kit (Novagen) or GST Purification kit (Pharmacia) according to the manufacturers' directions. Following elution from the affinity column, the protein preparation was dialyzed three times against 100 volumes of PBS. SDS-PAGE analysis of these samples showed them to be >80% pure by Coomassie blue staining (data not shown). One liter of initial culture yielded 2–5 mg of purified protein.

Rabbit antibody against GRAB was produced by Covance (Denver, PA). GST fusion protein containing the C-terminal 18 kDa of GRAB (amino acids 213–377) was used as antigen.

Protein Binding Assays

To test protein-protein interactions in HEK293 cells, indicated constructs were cotransfected into the cells using the calcium phos-

phate method. After 48 hr, cells were harvested and lysed in lysis buffer (50 mM Tris-HCl [pH 7.4], 50 mM NaCl, 2 mM EDTA, 0.5% Triton X-100, 1 mM DTT, 1.5 mM Na_3VO_4, 50 mM NaF, 10 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 5 μg/ml aprotinin, 1 μg/ml leupeptin, 6 μg/ml chymostatin, 0.7 μg/ml pepstatin, 1 mM phenylmethylsulfonyl Fluoride [PMSF]). Cell lysate was then centrifuged at 20,000 × g for 20 min to remove insoluble materials. Equal aliquots of the supernatant were incubated with Glutathione Sepharose 4B beads or protein A/G-agarose together with indicated antibody for 2 hr at 4°C and then washed four times with lysis buffer. Bound proteins were analyzed by SDS-PAGE followed by immunoblotting with indicated antibody as previously described (Luo et al., 1999). To get the maximal binding activity between Rab3A and GRAB, the endogenous nucleotide in cell lysate was removed by incubation in the presence of 10 mM EDTA, followed by gel filtration using a P6 column (Bio-Rad; Hercules, CA).

To test in vivo interactions between Rab3A and GRAB, adult rat brain was homogenized in lysis buffer and centrifuged at 20,000 × g for 20 min to remove insoluble materials. The resulting lysate was first precleared with rabbit IgG and protein G-agarose for 1 hr at 4°C and then incubated with rabbit anti-GRAB antibody coupled protein G-agarose for 2 hr at 4°C. The anti-GRAB antibody coupled protein G-agarose was prepared using an ImmunoPure Protein G IgG Plus Orientation Kit (PIERCE; Rockford, IL) following the manufacturer's instructions. Immunoprecipitated proteins were washed four times with lysis buffer, eluted in SDS sample buffer, and visualized by immunoblotting with mouse anti-Rab3A antibody or rabbit anti-GRAB antibody.

In vitro binding assays were performed using His6-tagged or GST-tagged fusion proteins, which were expressed and purified as described previously. Rab3A was purchased from Sigma. Indicated proteins were incubated with each other in lysis buffer for 30 min at 37°C followed by 2 hr incubation at 4°C. Samples were then precipitated with Glutathione Sepharose 4B beads or the indicated antibody combined with Protein A/G-agarose for1 hr at 4°C and then washed four times with lysis buffer. Bound proteins were analyzed by Western blot.

InsP6 Kinase Assay

InsP6 kinase enzymatic activity was assayed in 10 μl of reaction mixture containing 10 ng recombinant InsP6K1, 20 mM HEPES (pH 6.8), 6 mM $MgCl_2$, 1 mM dithiothreitol, 5 mM ATP, 5 mM NaF, 10 mM phosphocreatine, 0.01 mg/ml phosphocreatine kinase (Calbiochem), 5 μM InsP6, and 40 nM [^{3}H]InsP6. Samples were incubated at 37°C for 2 hr, and reactions were terminated either by addition of 1 μl 1M HCl or by immersion in an ice water bath (Saiardi et al., 1999). Assays of the activities of recombinant enzymes employed HPLC using a 4.6 × 125 mm Partisphere SAX column (Whatman Inc.; Clifton, NJ) that was eluted with a gradient generated by mixing Buffer A (1 mM Na2EDTA) and Buffer B (Buffer A plus 1.3 M $(NH_4)_2HPO_4$ [pH 3.8] with H_3PO_4) as follows: 0–5 min, 0% B; 5–10 min, 0%–30% B; 10–60 min, 30%–100% B; 60–75 min, 100% B. Fractions (1 ml) were collected and counted using 5 ml of Ultima-Flo AP LCS-cocktail Packard (Downers Grove, IL) (Saiardi et al., 2000).

Nucleotide Exchange Assays

The assays for dissociation of [^{3}H] GDP from Rab3A and binding of [^{35}S]GTP-γ-S to Rab3A were performed essentially as described (Kikuchi et al., 1995; Shirataki and Takai, 1995). The GDP displacement assay was initiated by preloading recombinant Rab3A (Sigma) with 0.5 μM [^{3}H]GDP, and the disassociation was triggered by adding 0.5 mM cold GTP either with 50 nM GST-GRAB protein or with control buffer. All reactions were carried out at 22°C in assay buffer (0.1 μg/ul BSA, 50 nM Rab3A, 20 mM Tris 7.6, 1 mM DTT, 5 mM $MgCl_2$, and 1 mM EDTA) and the aliquots were removed at the times indicated. Disassociation of [^{3}H]GDP was assayed by measuring the decrease in [^{3}H]GDP-Rab3A trapped on nitrocellulose filters (HAWP02500 membrane, Millipore; Bedford, MA). The binding of [^{35}S]GTP-γ-S to Rab3A was examined in the same assay buffer and at the same temperature as described (Kikuchi et al., 1995; Shirataki and Takai, 1995). Rab3A (50 nM) and GRAB protein (50 nM) were pre-incubated together in assay buffer at 37°C for 30 min, and the binding initiated by addition of 3 μM [^{35}S]GTP-γ-S (2 μCi/ml). Radiolabel associated with Rab3A was measured by filter assay.

Generation of Recombinant Adenovirus

InsP6K1 or GRAB cDNA was inserted into a pAdTrack-CMV vector (He et al., 1998). Recombinant viruses were generated, amplified, and purified using an AdEasy Adenovirus system (Quantum Technologies; Montreal, Canada) following the provided protocol. Viruses were used at moi values of 100 to infect cultured PC12 cells and 75%–80% infection efficiency was obtained routinely. Infection of PC12 cells was usually carried out the day before the GTP loading assay or the transmitter release assay.

GTP Loading Assay

PC12 cells were cultured on polylysine coated plates in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS), 5% horse serum, 5% glutamate, and 100 units penicillin-streptomycin at 37°C with 5% CO_2 atmosphere in a humidified incubator. Differentiation was initiated by 50 ng/ml nerve growth factor (NGF) with culture medium changed to DMEM with 2% horse serum and 1% FBS. After 7 days, the GTP loading assays were performed essentially as described (Rosen et al., 1994) with minor modification. PC12 cells were metabolically labeled in phosphate-free DMEM with 0.5 mCi/ml [^{32}P]H_3PO_4 for 4 hr at 37°C. After the labeling, cells were lysed in 0.5 ml of lysis buffer D (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM $MgCl_2$, 1% Triton X-100, and 4 μg anti-Rab3A antibody). Extracts were drawn ten times through a 0.22 gauge needle and mixed with 1:10 volume of PBS/1% bovine serum albumin/10% charcoal slurry and then rocked at 4°C for 30 min. After centrifugation for 5 min at 12,000 × g, the supernatant was mixed with 30 μl protein A/G-agarose and then incubated for 2 hr, followed by three washes with lysis buffer D and one with PBS. After the last wash, pellets were resuspended in 30 μl of 1 M KH_2PO4 (pH 3.4) and incubated at 85°C for 3 min. After centrifugation at 12,000 × g for 5 min, a 3 μl sample was spotted onto polyethyleneimine-cellulose TLC plates (EM Science; Gibbstown, NJ), and guanine nucleotides were resolved in 1 M KH_2PO4 (pH 3.4) for 2.5 hr. The TLC plate was exposed to film for 1 day at −70°C. Each GDP and GTP fractionation was quantitated using a Phosphorimager (Molecular Dynamics).

[^{3}H]Dopamine Release Assay

PC12 cells were cultured and differentiated as described previously, and [^{3}H]dopamine release was monitored out essentially as described (Sandberg et al., 1989; Steiner et al., 1996). PC12 cells treated with NGF for 7 days were incubated with [^{3}H]dopamine (NEN Life Science Products; Boston, MA) for 60 min prior to assay. Depolarization was initiated by addition of 40 mM KCl for 2 min at 37°C and stopped by adding ice-cold PBS-EDTA (10 mM) into cell medium. Cell supernatants were then centrifuged for 5 min at 12,000 × g, and radioactivity in the supernatant was measured.

Chromaffin Cell Culture and Human Growth Hormone (hGH) Release Assay

Primary bovine adrenal chromaffin cells were isolated and maintained as described previously (Holz et al., 1995; Wick et al., 1993). Twenty hours after plating, cells were cotransfected with the indicated plasmid and an expression vector containing hGH cDNA (pXGH, Nichols Institute; San Juan Capistrano, CA) using a GIBCO-BRL calcium phosphate transfection system kit following the manufacturer's instructions. hGH release was assayed 6 days after transfection as described (Holz et al., 1995; Wick et al., 1993). hGH release was stimulated by 20 μM 1,1-dimethyl-4-phenylpiperazinim (DMPP, a nicotinic agonist) during a 5 min incubation at 37°C. GH released into the medium and retained in the cells was measured using a radioimmunoassay for GH (Allegro hGH transient gene expression assay system, Nichols Institute). Secretion is expressed as the percentage of total hGH (hGH released into the medium and hGH retained in the cells).

Northern Blotting of mRNA

A multitissue poly(A)$^+$ RNA blot was purchased from Clontech (Palo Alto, CA). DNA probes were labeled by random priming to final specific activity circa 4 × 10^9 cpm/μg and used at 2 × 10^6 cpm/ml

in the hybridization reactions. GRAB and Rab3A probes were derived from the full-length GRAB and Rab3A, respectively. The β-actin control probe was supplied by Clontech (blotting data with control probe is not shown). Prehybridizations (2 hr) and hybridizations (16 hr) were carried out at 42°C in hybridization solution (1× Denhardt's, 50 mM Tris (pH 7.5), 1% SDS, 50% formamide, 1.0 M NaCl, 10 μg/ml sheared, denatured salmon sperm DNA). Following hybridization, three washes were performed at room temperature (RT) for 10 min each in 2× SSC, two for 30 min each at 65°C in 2× SSC supplemented with 0.5% SDS, and two for 30 min each at RT in 0.1% SSC. Following autoradiography, the membrane was stripped by incubating with 1% SDS in ddH2O at 90–100°C for 3–5 hr. Stripping was verified by overnight exposure to a PhosphorImager screen.

In Situ Hybridization

Fresh-frozen rat brain sections (2–3 month old, SD male) were fixed with 4% paraformaldehyde/PBS and permeabilized, prehybridized, and hybridized in 50% formamide, 5% SSC with 100 ng/ml unhydrolyzed digoxygenin-labeled probe overnight at 55°C. Sections were washed, blocked, and incubated overnight at 4°C in 4% normal goat serum in TBS with antidigoxygenin-AP antibody (Roche Molecular Biochemicals; Indianapolis, IN) at 1:5000. After washing in TBS, slides were developed in the dark with 1 ml of color development solution containing 3.375 mg/ml nitroblue tetrazolium, 3.5 mg/ml BCIP, and 0.24 mg/ml levamisole. The color reaction was allowed to run 48 hr at room temperature. The reaction was stopped in ddH2O, and the slides were sealed in Aquapolymount. Probes were generated from cDNA corresponding to ORF of GRAB or InsP6K1 subcloned in Bluescript II SK+ plasmid (Stratagene) by in vitro transcription using T7 or T3 RNA polymerases. Sense control probes used at equal concentration generated no specific signal.

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